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A Variant of Porcine Thyroxine-Binding Globulin Has Reduced Affinity for Thyroxine and Is Associated with Testis Size[1]

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ABSTRACT

The field of genomics applies the dissection of genetic differences toward an understanding of the biology of complex traits. Quantitative trait loci (QTL) for testis size, plasma FSH in boars, and body composition (backfat) have been identified near the centromere on the X chromosome in a Meishan–White Composite resource population. Since thyroid function affects Sertoli cell development and adult testis size in rodents, and thyroxine-binding globulin (*TBG*) maps to this region on the porcine X chromosome, *TBG* was a positional candidate gene for testis size. We discovered a polymorphism in exon 2 of the porcine *TBG* gene that results in an amino acid change of the consensus histidine to an asparagine. This single nucleotide polymorphism (SNP) resides in the ligand-binding domain of the mature polypeptide, and the Meishan allele is the conserved allele found in human, bovine, sheep, and rodent *TBG*. Binding studies indicate altered binding characteristics of the allelic variants of *TBG* with the asparagine (White Composite) isoform having significantly greater affinity for thyroxine than the histidine (Meishan) isoform. Alternate alleles in boars from the resource population are also significantly associated with testis weight. Therefore, this polymorphism in *TBG* is a candidate for the causative variation affecting testis size in boars.

developmental biology, follicle-stimulating hormone, mechanisms of hormone action, Sertoli cells, testis

INTRODUCTION

The unusual physiology of the highly prolific Meishan pig offers a unique opportunity to investigate factors that affect the onset of puberty and reproductive efficiency. Meishan pigs reach puberty at a much earlier age and produce larger litters, and boars have dramatically higher levels of circulating FSH and smaller testes compared with occidental breeds [1]. Reasons for these dramatic differences are not known, but investigators have developed resource populations using Meishan–White Composite crosses to dissect out the underlying genetic components of these physiological differences. Understanding the biology of these traits in males and females would allow for more efficient reproduction of commercial swine.

Testicular sperm production is positively related to testis size, and in an industry with a growing use of artificial insemination for production, the culling of boars because of reduced reproductive success is of economic concern. Pubertal plasma FSH and LH [2, 3] and mature testicular size are associated with genes encoded on the X chromosome of boars [2, 4], but the physiologic basis for differences in testicular development or circulating gonadotropins remains unknown. The attainment of adult testis size is a balance of germ cell, Sertoli and Leydig cell, proliferation, differentiation, and apoptosis that is critical for normal sexual development, sperm production, and fertility, but number of Sertoli cells is a primary determinant of testicular size [5, 6]. Many hormonal and regulatory stimuli that control the development of these testicular compartments have been identified, and several models exist to test various endocrine and physiological paradigms.

Thyroid hormones play a critical role in the normal development of the mammalian testis [7–9]. Transient hypothyroidism during the neonatal period of testicular growth in rodents results in an extended period of Sertoli cell proliferation, a dramatic increase in mature testicular size [10–12], and a reduction of plasma FSH in males of about 50% [13]. In boars, this sensitive period for Sertoli cell proliferation appears to occur before birth due to the inability of unilateral castration on the first day of life to produce a significant increase in Sertoli cell number at maturity [6]. McCoard et al. [14] predicted that differences in availability of triiodothyronine (T3) to developing fetuses might account for differences in mature testicular size in boars. Meishan male fetuses had greater plasma concentrations of total and free T3 and a greater amount of bound T3 in their plasma than White Composite male fetuses from 75–105 days of pregnancy. Higher neonatal thyroid hormone levels are also associated with smaller testes in bulls [15].

The gene for thyroxine-binding globulin (*TBG*) resides within the quantitative trait loci (QTL) for testis size and plasma FSH on the porcine X chromosome [16, 17] and within a QTL for backfat [18–20]. *TBG* is a 54 kDa glycoprotein of hepatic origin and the principal transport protein of thyroid hormones in serum. In addition to transporting T3 and plasma thyroxine (T4), the potential to regulate availability of thyroid hormones within tissues exists through proteolytic cleavage of *TBG* [21]. Thyroid hormones play a critical role in regulating the growth, development, differentiation, and metabolism of virtually all tissues [22].

Therefore, *TBG* presents itself as a positional candidate gene for testis size in the boar. We hypothesize that polymorphisms in *TBG* cause decreased circulating free thyroid hormone levels and an extended proliferation of Sertoli cells resulting in larger testes in White Composite boars.

[1]The nucleotide sequence data reported in this paper has been submitted to GenBank and has been assigned the accession number AY550250. Mention of trade names or commercial products is solely for the purpose of providing information and does not imply recommendation, endorsement, or exclusion of other suitable products by the U.S. Department of Agriculture.

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TABLE 1. Primers used for sequencing and genotyping porcine TBG.

Forward	Primer sequence	Reverse	Primer sequence	Size (bp)	Product
608F	tcccagacctcaaaccaaac	854R	aggacaaagagtgccagagc	1326	intron 1
687F	tccgcccaagacagaagagg	1022R	tcctgaatgcccatcttca	1056	intron 2
974F	ctgccacatatgaccttgga	1331R	tcccattgcaataagcacac	956	intron 3
37F	ccttccaaaatgccactgtt	423R	ttccagctccttctttggaa	387	exon 1
244F	ttttcccctgtgagcatttc	721R	agcaccccaccaaattattg	478	exon 1
1675F	tgatggaaaagttcacattgga	2073R	tctcatctgtgccattatgc	399	exon 2
2669F	gacagagagggagggaatca	3016R	atctggcgtaggaggagtca	348	exon 3
3366F	tcaacacaatacagaaaagggcta	3723R	ttgcaataagcacacgcaat	358	exon 4
Genotyping primers					
1022RTUS5	agcggataacaatttcacacagggaaggacaaagagtgccagagc	US5	biotin-agcggataacaatttcacacagg		
Probe	cagatggaacaatactat				

MATERIALS AND METHODS

Resource Population

Animals used were from generations 8 and 10 (F8 and F10) produced from the original resource population [3, 23] used to identify QTL and consisted of three-fourths White Composite × one-fourth Meishan. Testis weights and genotypes were obtained on 84 F8 boars; 26 were bilaterally intact, and 31 and 27 were unilaterally castrated at 25 or 56 days of age, respectively, after induction of local anesthesia [2]. At 10 mo of age, all boars were given general anesthesia [2], after which remaining testes were removed and weighed. Blood was collected at 120 and 300 days of age from the 84 F8 boars for determination of plasma FSH and total T3 and T4; free T4 and T3 uptake were determined on a subsample of 40 F8 boars. Serum obtained at 220 days from 61 F10 boars and 145 F10 gilts was pooled within the sire family for *TBG* measurements; plus, serum concentrations of total T3 and T4 and T3 uptake were evaluated on a subsample of 34 F10 boars. Procedures for the handling of animals complied with those specified in the *Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching* (Savoy, IL: Federation of Animal Science Societies; 1999).

PCR and Sequencing

Primer pairs for amplification of *TBG* from genomic DNA were designed from porcine sequences deposited in GenBank (Table 1) using primer 3 [24] (code available at http://www-genome.wi.mit.edu/genome_software/other/primer3.html). PCR was performed in a PTC-225 DNA engine (MJ Research Inc., Watertown, MA) using 0.25 U Hot Star *Taq* polymerase (Qiagen, Valencia, CA); 1× of supplied buffer; 1.5 mM $MgCl_2$; 200 μM dNTPs; 0.8 μM each primer; and 100 ng of genomic DNA in 25 μl reactions. Five microliters of the PCR reaction was electrophoresed in 1.5% agarose gels to determine quality of amplification, and the remainder was prepared for sequencing [25]. Chromatograms were imported into the MARC database, bases were called with Phred and assembled into contigs with Phrap, and polymorphisms were identified using Polyphred and assessed using Consed [26].

SNP Genotyping

SNPs were mapped using a primer extension assay on the Sequenom MassArray system (San Diego, CA). Fifty microliters of PCR reactions contained 100 ng of genomic DNA, 0.25 U HotStar *Taq*, 1× of supplied buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.4 μM forward primer (687F), 0.04 μM tailed reverse primer (1022RTUS5), and 0.25 μM biotinylated tail primer US5 (Table 1). The primer extension reaction used 10 μM of probe primer (Table 1).

Radiation Hybrid Mapping

Genes were mapped using the 118-clone INRA–University of Minnesota porcine radiation hybrid (IMpRH) panel [27]. Primers used were described above or designed from intron sequences obtained with the original amplification primers. Amplifications were performed in 15 μl PCR in duplicate using 25 ng panel DNA, 1.5 mM $MgCl_2$, 200 μM dNTPs, 1 μM each primer, 0.25 U Hot Star *Taq*, and 1× of supplied buffer. The PCR mixture was held at 94°C for 15 min and cycled 40 times at 94°C for 20 sec, the indicated annealing temperature for 30 sec, and with extension at 72°C for 1–1.5 min, followed by a final extension at 72°C for 5 min. One half of the reaction was loaded on 2% agarose gels and manually genotyped. Data were analyzed for two-point and multipoint linkage with the IMpRH mapping tool and submitted to the IMpRH database (http://imprh.toulouse.inra.fr/). Carthagene (http://www.inra.fr/bia/T/CarthaGene/) was used to estimate multipoint marker distance and order using all public markers [28] on the X chromosome in the IMpRH database (http://imprh.toulouse.inra.fr/) and those developed in this study to approximate the position of RH mapped markers on the MARC linkage map. Markers developed in this study have been submitted to the IMpRH public database.

Linkage Analyses

Linkage analyses were performed as described by Rohrer et al. [29] where TWOPOINT analyses were used to indicate the chromosome linkage group and the ALL, FLIPS, and FIXED options were used to determine the multipoint position of the marker (CRIMAP v2.4). Multipoint locations for all mapped markers are based on the latest published swine genetic map (http://www.marc.usda.gov/) [29].

Plasma Hormone Measurements

Plasma thyroxine (T4) and triiodothyronine (T3) concentrations were determined within a single assay with antibody-coated tube RIA kits (DSL, Webster, TX) [14]. Coefficients of variation were <5% for two serum pools that were included in the T4 assay and were <12% for these pools in the T3 assay. Free T4 was measured by enzyme immunoassay (EIA) (DSL). Triiodothyronine (T3) uptake was determined in a single assay using T3-antibody–coated tubes (ICN Pharmaceuticals, Orangeburg, NY); the reference preparation had a mean activity of 36.4% with a coefficient of variation of 4.7%. Plasma FSH concentrations were determined as a single assay [30]; coefficients of variation ranged from 10% to 17% for four serum pools that were included in the assay.

T4 Binding to Serum TBG

The method used to measure *TBG* binding was a modification of that previously described [31, 32]. Serum samples were diluted 1:400 in 0.075 M Na-barbital buffer, pH 8.6. One milliliter diluted samples were incubated for 1 h at 37°C with 10 000–20 000 cpm of ^{125}I-T4 and 0–200 nM unlabeled T4 to measure total binding, along with parallel tubes containing 100-fold excess unlabeled T4 to measure nonspecific binding. After incubation, tubes were quickly chilled in an ice bath for 20 min before the addition of 0.5 ml (250 mg of prewashed resin beads in H_2O) of Dowex AG-1-X8 anion exchange beads (Bio-Rad Laboratories, Hercules, CA) to separate bound and free hormone. The tubes were mixed on ice for 5 min three times, and 1 ml of ice-cold H_2O was added to each tube. The samples were centrifuged for 2 min at 1500 × *g* at 4°C and maintained on ice, and duplicate samples of 1 ml were removed and counted to estimate bound hormone. The remainder of sample with resin beads was counted to determine total counts of each sample. Percent binding of each sample was calculated, and the concentration of binding was determined by the amount of hormone added to each tube. Nonspecific binding was subtracted from total binding to estimate specific binding for each sample. Data were plotted, and dissociation constants (Kd) and binding capacity (Bmax) were determined according to Scatchard [33]. Single-point assays of individual animals were diluted and assayed as described at saturating concentrations (200 nM) of T4.

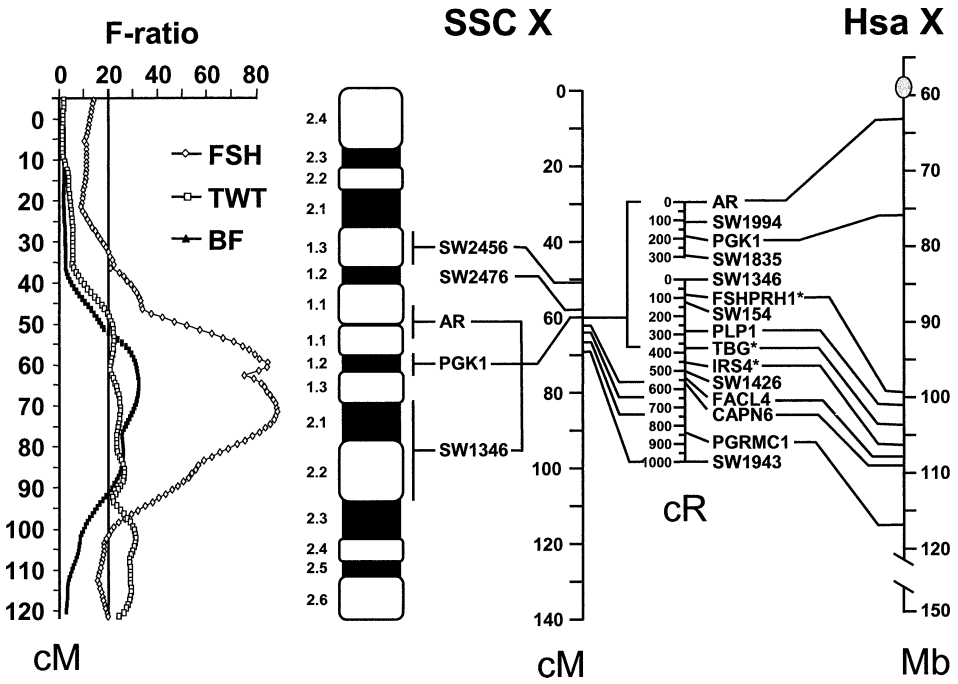


FIG. 1. F-ratio curves of associations of X chromosome QTL aligned to selected markers of the porcine cytogenetic, genetic, and physical maps compared with the human physical sequence map. Units are in centimorgans (cM), centirays (cR), and megabases (Mb). New gene assignments on the physical map are shown with an asterisk. Markers that map to the same position on the genetic map (AR to *TBG*) are grouped with a bracket. Full marker descriptions and the complete current linkage map can be found at http://www.marc.usda.gov/.

Heat Denaturation of TBG

Serum samples were diluted 1:50 in phosphate-buffered saline (PBS), 150 mM NaCl, 10 mM phosphate buffer, pH 7.4, and heated at 55°C in a water bath for 0–40 min [32]. Samples were then chilled on ice and centrifuged for 15 min at 13 000 × *g* and 4°C to remove denatured proteins. Samples were further diluted 1:4 in Na-barbital buffer, as above, and assayed for residual *TBG* binding. *TBG* binding was then calculated as percent binding compared with similarly diluted samples that were maintained on ice. The half-lives of heat denaturation were calculated by the regression coefficient of semilogarithmic plots of residual T4-binding versus time of incubation.

Statistical Analyses

Statistical associations of *TBG* genotype with phenotypes were evaluated with SAS PROC GLM (SAS, Cary, NC) in 84 F8 generation boars with 10-mo testes weight and serum FSH concentrations measured. The model fitted for testes weight included *TBG* genotype and treatment as fixed effects and body weight as a covariate. The model fitted for serum FSH was similar except that body weight was not included. Treatment was age at unilateral castration. *TBG*-binding and plasma hormone data were evaluated statistically by mixed model procedures of SAS [34]. The model for testicular and body weight, plasma FSH, T3 and T4 concentrations, and T3 uptake included fixed effects of age at unilateral castration, X chromosome QTL, and the interaction of these; sire was the random effect. Age at removal of the left testicle was a covariate. The mode for TGB binding affinity included the fixed effect of the X chromosome QTL with assay as the random effect. A repeated measures analysis was used for free T4 measurements on the 40 F8 boars. Data are reported as least squares means and standard errors.

RESULTS

Physical Gene Map of QTL Region

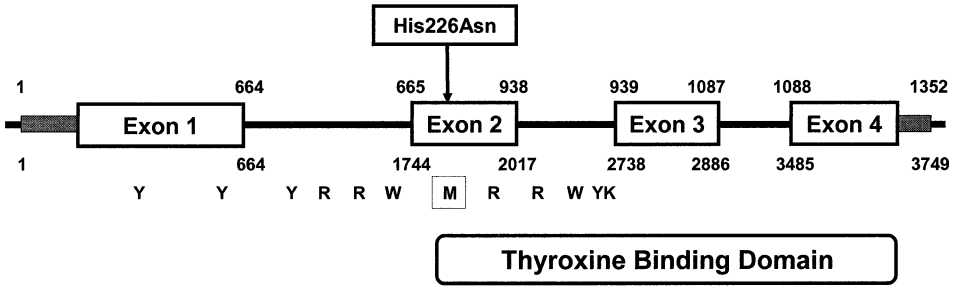
TBG maps within the QTL interval for testis size, plasma FSH, and body composition [16] in a region that has a low level of recombination. The comparative physical map demonstrates that the equivalent of about 40 Mb of the porcine X chromosome does not recombine (65–105 Mb on the human X chromosome; Fig. 1). *TBG*, FSH primary response homolog of rat (*FSHPRH1*), and insulin receptor substrate 4 (*IRS4*) were mapped as new assignments on the IMpRH panel. Genes in this region share the same genetic position at 60 cM on the porcine map. Of the genes mapped on the IMpRH panel in this region, (i.e., *AR*, *PGK1*, *FSHPRH1*, *PLP1*, *TBG*, *IRS4*, *FACL4*, *CAPN6* and *PGRMC1*), no rearrangements were identified (Fig. 1), suggesting that the physical distance and gene order is similar in the pig as in human.

Sequencing of Porcine TBG

The porcine *TBG* gene was sequenced using exon primers (Table 1) designed from the full-length TIGR contig TC110296 found in the TIGR porcine gene index (http://www.tigr.org). Gene organization and intron size were very similar to the human *TBG* gene (Fig. 2) and, except for a small segment (115 base pair) of a repetitive sequence in intron 3, the complete porcine sequence aligned with 78% nucleotide identity to the human gene. Primers were then designed from flanking cDNA and intron sequences to screen the complete coding region [32] and splice junctions for polymorphisms (Table 1). By sequencing 40 parents from the MARC resource populations and eight parents of the MARC mapping family, eight SNPs were found in introns 1 and 2, two silent SNPs in exon 1, one silent SNP in exon 2, and a nonconservative A/C polymorphism in codon 226 was identified in exon 2 that changes the consensus histidine to an asparagine (Fig. 2). The consensus C allele was found to be Meishan-specific. No other polymorphisms that changed an amino acid or were located near splice sites or lariat branch-points in the introns were found. The frequency of the A and C alleles in the boars used in this study was 0.71 and 0.29, respectively, and 0.65 and 0.35 in gilts, respectively.

Testis Weights and Plasma FSH

Total testicular weight at 300 days was significantly heavier in boars from the F8 generation carrying the A allele than in boars with the C allele of *TBG* ($P < 0.001$; Fig. 3). Body weight was not influenced by QTL ($P > 0.1$). Intact boars had greater total testicular weight than unilaterally castrated boars ($P < 0.001$); there was no difference in total testicular weight of boars castrated unilaterally at

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WC pig MMHQMEQYYNLVDTELNCTV
MS pig MMHQMEQYYHLVDTELNCTV
human  MMHQMEQYYHLVDMELNCTV
bovine MMHQMEQYYHLVDTELNCTV
sheep  MMHQVDQYYHLVDTELNCTV
rat    MMHQLEQYYHYVDVELNCTV
mouse  MMHQLEQYYHYVDMELNCTV
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FIG. 2. Gene organization of porcine *TBG* and the location of SNPs. Coding regions of exons are shown boxed. Numbering above exons reflects position in mRNA, and numbers below indicate position on genomic DNA. SNPs are indicated by their IUB code with the position of the His226Asn SNP shown above exon 2. The binding domain is indicated below. Below the gene structure is a partial alignment of the amino acid sequence in exon 2 from the species indicated. The consensus histidine and the amino acid change is underlined in bold.

25 or 56 days of age ($P > 0.3$), and there was no interaction of genetic alleles with unilateral castration ($P > 0.18$). Plasma FSH concentrations were significantly greater at 300 days of age in boars with the C allele than in boars with the A allele (1.04 ± 0.06 vs. 0.83 ± 0.08, respectively; $P < 0.02$).

Association tests at each marker in the QTL interval, ranging from 51 to 70 cM on the porcine linkage map, indicate that *TBG* at relative position 60 cM is the most significant marker ($P < 0.001$) associated with both testis size and FSH in F8 boars (Fig. 4).

Thyroxine Binding to Serum TBG

A single high-affinity saturable binding site was demonstrated for thyroxine in diluted porcine sera (Fig. 5). The average binding affinity (Kd) was 37 nM and the average total binding sites (Bmax) was 346 nM (Fig. 6). The binding affinity for serum pools of boars (three to six littermates; n = 5 replicates) with common sires was significantly greater for hemizygous animals with A genotypes than C genotypes (29.0 ± 3.63 vs. 43.7 ± 3.63, $P < 0.02$; Fig. 7). The concentration of *TBG* in serum, or total number of binding sites measured in single-point assays at saturating concentrations of 200 nM T4, was not different between the A and C genotypes (333.1 ± 42.38 nM vs. 334.5 ± 41.79 nM, respectively). The binding affinity for gilts averaged 44.8 nM, and Bmax was slightly higher than values found in boars at 515 nM. The same pattern for binding affinities was seen for homozygous AA and CC serum pools from females (30.9 ± 2.11 nM vs. 55.3 ± 1.72 nM for AA vs. CC females, respectively; n = 2 replicates) and pools of heterozygous females were intermediate in their binding affinity (48.2 ± 1.33 nM; n = 3; Fig. 7).

Heat Stability of T4 Binding to TBG

The residual binding of T4 to serum heated at 55°C was similar for A and C isoforms of *TBG* (half-lives approximately 17 and 13 min, respectively; Fig. 8).

Thyroid Hormone Levels

Total serum T3 and T4 levels were not different in boars at 220 days with differing *TBG* genotypes (1.18 ± 0.063 vs. 1.17 ± 0.084 ng/ml and 34.5 ± 1.28 vs. 33.4 ± 1.71 ng/ml for T3 and T4 for boars with A and C genotypes, respectively). Free T4 was lower in boars with the A allele

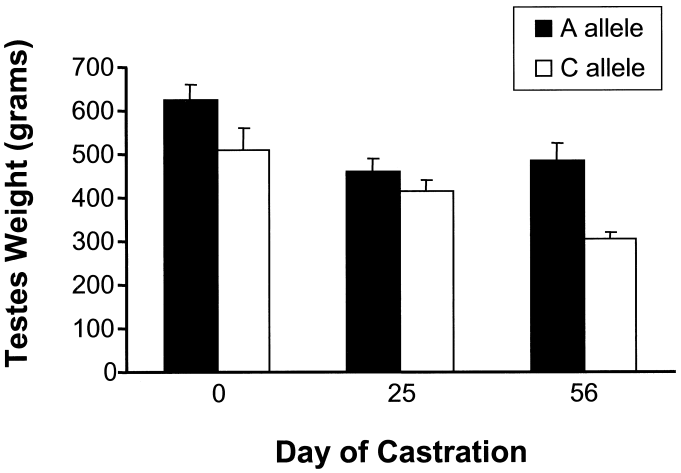

FIG. 3. Testicular weights of F8 boars at 300 days of age carrying alternate alleles of *TBG* that were either left intact or castrated at days 25 or 56. Total testicular weight was significantly heavier in boars with the A allele than in boars with the C allele of *TBG* ($P < 0.001$).

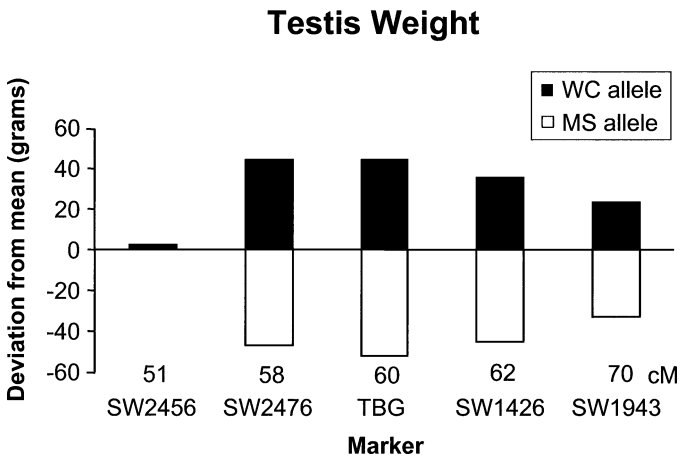

FIG. 4. Association of X chromosome marker genotypes with testicular weights. Data are shown as deviation from the mean testes weights in grams and association with either the White Composite (WC) or Meishan (MS) allele for that marker. Marker position in centimorgans (cM) is shown below.

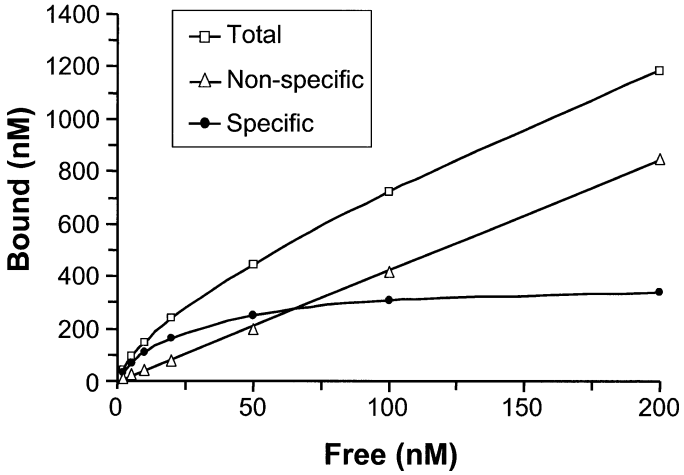

FIG. 5. Binding curves of ^{125}I-thyroxine binding to porcine *TBG*. Pooled boar serums were diluted and incubated with ^{125}I-T4 at 37°C for 1 h at the final concentration of T4 indicated. Nonspecific binding tubes contained 100-fold excess of unlabeled hormone. Bound and free were separated using Dowex AG-1-X8 anion exchange resin.

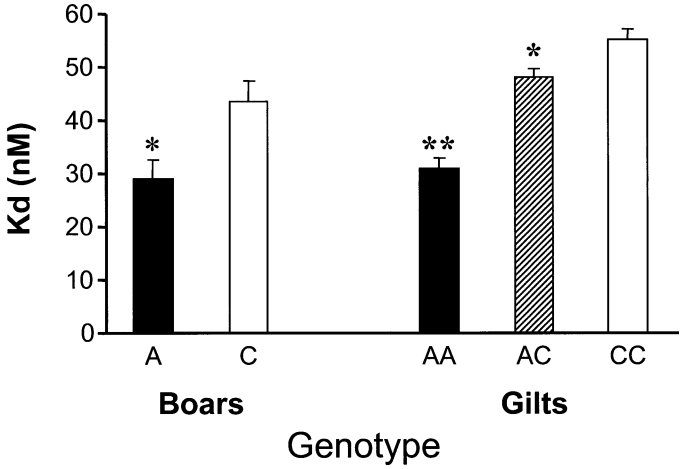

FIG. 7. Mean *TBG* binding values of four separate assays of littermate pools of boars with alternating *TBG* alleles and two separate assays of gilts representing three genotypes. Dissociation constants of boars determined by Scatchard analysis were significantly lower for the A than C allele ($P < 0.02$). In gilts, the AA genotype differed from the other two genotypes in binding affinity ($P < 0.001$), and AC differs from CC ($P < 0.02$).

than C allele (6.5 ± 0.3 pg/ml vs. 7.6 ± 0.3 pg/ml, respectively; $P < 0.02$; Fig. 9), and T3 uptake was lower in boars with the A allele than with the C allele (33.88% ± 0.41% vs. 38.02% ± 0.41%, respectively; $P < 0.001$; Fig. 9).

DISCUSSION

Variation in testis size of boars is due, in part, to the effects of genes located near the centromere on the X chromosome [3]. Because of extensive evidence for the role of thyroid hormones in testicular development and ultimate testis size, thyroxine-binding globulin is a physiological and positional candidate gene for determination of adult testis size. Due to lack of recombination in this region of the porcine X chromosome, traditional fine-mapping approaches cannot be used to further narrow the QTL region in these populations. The single nonsynonymous polymorphism discovered in the coding region of the boars used in this study was associated with altered function of the mature protein and supports the idea that different isoforms of *TBG* may affect testis size. Because of a substantial increase in affinity of *TBG* for T4 (50%) in White Composite pigs, a proportional decrease in availability of thyroid hormone to target tissues would be expected. This result is supported by the lower circulating free T4 and T3 uptake measurements in White Composite boars. Since T3 uptake indirectly estimates the amount of *TBG* in a sample, lower T3 uptake in animals carrying the A allele is indicative of their higher affinity for thyroid hormone. As in other mammals, circulating levels of thyroid hormones in the pig are low during the prenatal period, increase dramatically at birth, peak during the neonatal period, and rise again near puberty [35]. *TBG* levels follow a similar pattern [14]. Since most of the circulating T4 is bound to *TBG*, and because of the stronger affinity of *TBG* for T4, changes in affinity would have large effects on free T4 hormone concentrations [21] but would not be reflected in measurements of total circulating thyroid hormones or free T3.

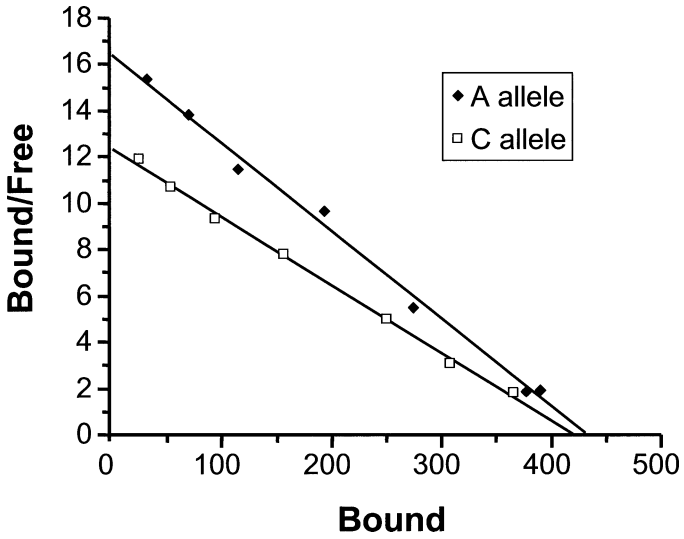

FIG. 6. Scatchard plots of binding data from representative littermate pools of boars with alternating alleles of *TBG* (R^2 = 0.9941 and 0.9971 for the A and C alleles, respectively). Each pool represents six boars. Dissociation constants in this experiment were 26.4 and 34.1 nM for the A and C alleles, respectively.

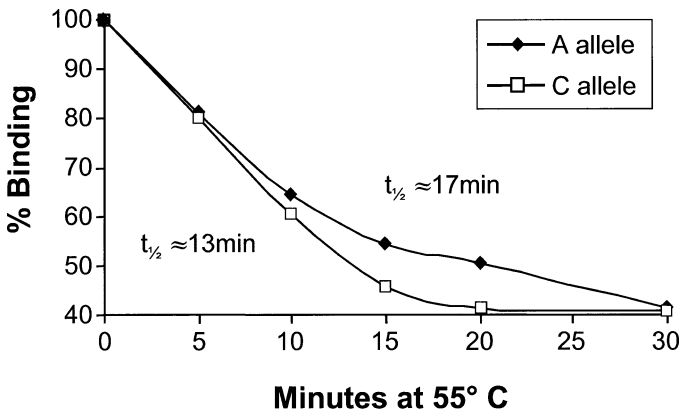

FIG. 8. Residual *TBG* binding activity after heat denaturation at 55°C. Data are expressed as binding activity remaining relative to initial values. Each curve represents the mean of four separate determinations. Half-lives of *TBG* in serum from pools of boars with the A or C alleles were similar, $t_{½} \approx 17$ and 13 min, respectively ($P = 0.1488$).

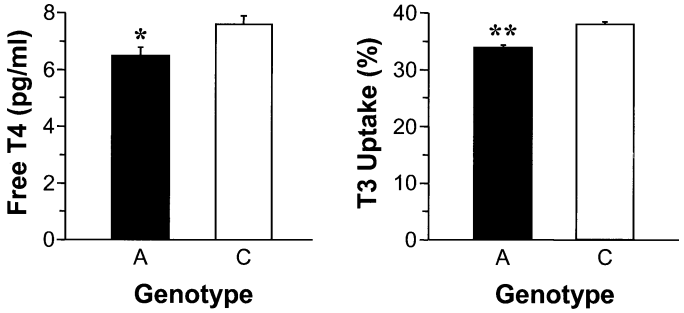

FIG. 9. Free T4 and T3 uptake values of F8 boars having A or C genotypes (n = 20/group; $P < 0.02$ and $P < 0.001$, respectively).

Several allelic variants of human *TBG* have been identified, resulting in partial or complete *TBG* deficiency, but the patients are euthyroid [36]. These mutations usually alter the binding characteristics, stability, or secretion of the mature protein [37–40]. One amino acid substitution (Ile96Asn) in human *TBG* results in an additional carbohydrate chain, impaired secretion, increased degradation, and decreased affinity for T4 [40]. The substitution of Asn for His in the pig resides in a hydropic fold of the hormone-binding site of the protein, and substitution of a charged, aromatic amino acid (His) with a small, polar amino acid (Asn) with different solvent and hydrophobicity properties could conceivably alter the structure of the protein. Histidine is an amino acid most often found in active and protein binding sites and is generally not very tolerant of substitutions. An in vitro construction of a human variant of *TBG*, substituting the adjacent amino acid, leucine 227, for proline, resulted in an isoform with impaired secretion from *Xenopus* oocytes and lacking detectable T4 binding [41]. The characterization of these variants support the functional differences observed in the porcine *TBG* isoforms reported here.

Previous studies designed to influence testis size in boars by inducing transient hypothyroidism at an early age have failed [42, 43]. One possible reason for this is that the treatment period was initiated too late in Sertoli cell proliferation to have effects on adult testis size, since the period of maximal cell proliferation occurs prenatally in boars [44] and testicular size of boars appears to be a function of the duration of Sertoli cell proliferation [6]. It is also possible that the boars in previous studies likely have the A allele, and the higher affinity form of *TBG* and hypothyroidism does not further reduce the amount of available hormone to the tissues or cause a change in testicular size. The consensus amino acid among all species with available sequence is histidine, and it seems probable that the asparagine substitution underwent selection, possibly on the basis of testis size. Efforts to prove that this polymorphism causes an increase in testis size will be difficult. The consensus allele was only found in Chinese pigs, so comparisons among breeds will be uninformative; all breeds with the A allele have larger testes [45]. Sequencing of related Suidae species could determine if the approximate occurrence of the mutation corresponds to important periods of domestication of swine. Because this region of the X chromosome does not recombine, it is not possible to introgress this allele into other populations and determine the effect on testis size without the introduction of a large number of other genes being included. Efforts to disrupt the association of *TBG* with surrounding genes on the X chromosome in several generations of our resource population were unsuccessful. An experiment to transgenically introduce the alternate allele into these breeds of pigs could address this problem once a method to modify targeted regions of the porcine genome become routine.

A QTL for FSH with a large effect has also been identified in this region (Fig. 1). In addition to causing a large increase in testis size, neonatal hypothyroidism in the rat is associated with a permanent reduction in pituitary FSH [13]. Meishan boars have several-fold greater circulating FSH levels than conventional breeds [1, 3, 45], and because these QTL regions overlap, *TBG* is a candidate for this QTL as well. It is possible that this QTL region is represented by more than one QTL. The evaluation of additional markers and construction of haplotypes should allow further study of these chromosomal regions and the genes involved in these biologically relevant reproductive traits.

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